Parent-of-origin has no detectable effect on survival days of Marek's disease virus infected White Leghorns

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ABSTRACT Marek's Disease (MD) is a neoplastic disease of chickens and remains as a chronic infectious disease that threatens the poultry industry. Improving genetic resistance to MD in poultry is an important long-term goal, which would significantly augment the current control measures against MD and eventually reduce the annual economic loss. In this study, survival patterns of F_2 birds from 2 reciprocal crosses were compared to examine possible difference in survival between the reciprocal crosses in response to MD virus (MDV) challenge. A total of 246 and 224 F_2 chicks derived from reciprocal crosses of lines $6_3 \times 7_2$ and lines $7_2 \times 6_3$, respectively, were sampled from an MDV challenge trial and survival days were recorded from the MDV-inoculation date to the end of experiment. Statistical analyses, including Principal Component Analysis (PCA) followed by a cox-regression model, showed there was no significant difference in survival days between reciprocal crosses ($P > 0.05$). To the best of our knowledge, this is the first MD survival study on reciprocal crosses of 2 genetically diversified lines of chickens differing in MD resistance. This report documented the experimental evidence that the genetic lineage of grandparental (maternal or paternal) effect on survival days was minimal, if present at all.

Key words: reciprocal crosses, grandparental effect, Marek's disease, survival analysis

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INTRODUCTION

In genetics, a reciprocal cross is a pair of crosses between males of 1 genetic line and females of another genetic line with phenotypes that differ, and vice versa. Reciprocal crosses are designed to test the role of parental sex on a given inheritance pattern, and both parental lines must be true breeding. In 1 cross, a male expressing the trait of interest will be crossed with a female not expressing the trait or expressing the trait in a significantly different way, and vice versa in the other cross. Such types of experiments are different to those that are carried out in the contemporary era of next generation sequencing. Given that a trait of interest is either autosomal or sex-linked and in combination with either complete dominance or incomplete dominance, the F_2 populations of a reciprocal cross will reveal the mode of inheritance of a trait of interest. Sex linkage was first reported by Doncaster and Raynor (1906), who studied the inheritance of a color mutation in a moth *Abraxas grossulariata*. Reciprocal crosses did not infrequently produce different results (Dobzhansky, 1935), but sex-linked reciprocal crosses were usually different from each other (Phillips, 1913). Mendel has already referred and verified that generally there are no inheritance differences between reciprocal crosses, except sex-linked and cytoplasmic inheritance (Mendel, 1965). However, the impact of parent-of-origin on disease resistance in poultry is yet unknown.

Survival analysis (Binet et al., 1981; Miller Jr, 2011; Harrell, 2015) is a useful and effective approach to identify the differences between reciprocal crosses in relation to disease resistance. It is a statistical method for analyzing longitudinal data on the occurrence of events, including death, injury and onset of illness, recovery from illness (binary variables) or transition above or below the clinical threshold of a meaningful continuous variable. And it also accommodates data from randomized clinical trial or cohort study design. The main purpose of survival analysis is to model the underlying distribution of the failure time variable and

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to assess the dependence of the failure time variable on the independent variables. It compares mean time-to-event between 2 groups using a *t*-test or linear regression or compares proportion of events in 2 groups using risk/odds ratios or logistic regression (Hosmer et al., 2013). Survival data refers to a variable, which measures the interval between a particular start point and a particular end point of interest and it consists of a response (event time, failure time or survival time) variable that measures the duration of time until a specified event occurs and possibly a set of independent variables thought to be associated with the failure time variable (Klein & Moeschberger, 2005).

Marek's disease (**MD**) is a lymphoproliferative disease of domestic chickens caused by Marek's disease virus (**MDV**), an oncogenic and highly contagious α-herpesvirus. Marek's disease has been controlled by vaccination but sporadic outbreaks of MD almost routinely occur in some parts of the world including United States (Biggs & Nair, 2012). Genetic resistance is commonly considered as an attractive approach to augment the current control measures against MD (Bacon et al., 2001; Cheng et al., 2008; Yu et al., 2011). There are 2 highly inbred chicken lines 6_3 and 7_2 that have been established and maintained since 1939 in USDA-ARS, Avian Disease and Oncology Laboratory (**ADOL**) at East Lansing, Michigan (Stone, 1975). They are 2 of many unique experimental chicken lines at ADOL, which are recognized as important genetic resources for various projects primarily focusing on tumor-virus induced diseases in poultry. The line 6_3 is resistant to MD, while the line 7_2 is susceptible (Bacon et al., 2000). Several differentially expressed genes and different genotypes were identified in our former transcriptome study (not published) and also in Cheng's study (Cheng et al., 2015) between 2 reciprocal crosses of F_1 White Leghorns in response to MDV infection, but no evidence for a phenotyping study between reciprocal crosses was reported. In the present study, therefore, survival days of 2 groups of F_2 White Leghorns, derived from a reciprocal cross of the lines 6_3 and 7_2, were generated and examined post a very virulent plus (vv+) strain of MDV challenge to explore the inheritable impact of parent-of-origin effect on MD progression.

MATERIALS AND METHODS

Chickens

The highly inbred line 6_3 and 7_2 chickens were sampled for a reciprocal cross, $6_3 \times 7_2$ and $7_2 \times 6_3$. The F_1 birds were intermated per cross to produce the F_2 groups. A total of 246 F_2 chicks were produced from the F1 of the $6_3 \times 7_2$ cross, and 224 F_2 chicks, from the F_1 of the $7_2 \times 6_3$ cross.

Infection Experiment

A partially attenuated vv+ MDV, 648A passage 50 (Witter et al., 2005) was used to inoculate the birds intra-abdominally at day 5 post hatch at a dosage of 500 plaque-forming units (PFU) per chick. Marek's disease virus-challenge trial was conducted in the BL-2 facility at ADOL. All the experimental birds were incubated and observed daily at the same time. Any bird losing mobility due to disease progression at any time point during the experimental period was removed from the isolator and euthanized for necropsy. All experimental birds surviving the full experimental period were euthanized 8-wk post infection. Survival days of each bird were recorded. Survival days was defined as the number of calendar days between the date when the experimental birds were inoculated with MDV and the date when a bird died (none censored) or the date when the surviving experimental birds were euthanized at the end of the experiment (censored). All experimental chickens were managed and handled according to the guidelines established and approved by the ADOL Animal Care and Use Committee (ACUC) (April 2005).

Other Data Collection

At necropsy, each experimental bird was also identified and recorded for binary variables including specific reciprocal cross ($6_3 \times 7_2$ or $7_2 \times 6_3$), sex (M or F), MD (Yes or No, true for the rest of binary variables), gonadal tumor, heart tumor, intestinal tumor, kidney tumor, liver diffusion, liver focal, liver small, liver medium, liver large, liver tumor, lung tumor, pancreas tumor, spleen diffusion, spleen focal, spleen medium, spleen large, spleen tumor, and 5 ordinal categorical variables—-bursa atrophy, thymus atrophy, brachial, sciatic, and vagus nerve enlargement (coded as 0, 1, 2, 3, and 4, representing normal, minor, medium, severe, and very severe atrophy or enlargement, respectively).

Data Analysis

To screen the group of categorical variables, other than reciprocal cross, sex, and MD, for contributable effect on survival days, we first conducted a principal component analysis (**PCA**) using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) to identify the major relevant variables to include in the survival analysis model along with reciprocal cross, sex, and MD. There are several methods commonly used for survival data analysis, which include Kaplan–Meier (Kaplan & Meier, 1958), life tables (Cox, 1992), Log-Rank test (Mantel, 1966), and Cox's proportional hazards regression model (cox-regression model) (David, 1972; Cox, 1975). The cox-regression model was used in the survival data analysis to encompass all the selected dependent variables (reciprocal cross, sex, and MD) and covariates (bursa atrophy, thymus atrophy, brachial, sciatic, and vagus nerve enlargement) using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). We considered a P-value < 0.05 to be of statistical significance.

Table 1. Principal Component Analysis statistics.

Variable	Eigenvalue	Difference	Proportion	Cumulative proportion
Vagus	5.43	2.49	0.25	0.25
Thymus atrophy	2.94	0.71	0.13	0.38
Bursa atrophy	2.23	0.63	0.10	0.48
Brachial	1.59	0.20	0.07	0.55
Sciatic	1.40	0.10	0.06	0.62
Spleen tumor	1.29	0.30	0.06	0.68
Heart tumor	0.99	0.05	0.05	0.72
Liver tumor	0.95	0.02	0.04	0.76
Spleen diffusion	0.93	0.21	0.04	0.81
Spleen large	0.72	0.02	0.03	0.84
Liver focal	0.70	0.05	0.03	0.87
Spleen medium	0.65	0.09	0.03	0.90
Liver large	0.56	0.04	0.03	0.93
Spleen focal	0.52	0.12	0.02	0.95
Kidney tumor	0.40	0.05	0.02	0.97
Liver medium	0.35	0.14	0.02	0.98
Liver diffusion	0.21	0.08	0.01	0.99
Gonadal tumor	0.13	0.11	0.01	1.00
Lung tumor	0.01	0.01	0.00	1.00
Intestinal tumor	0.00	0.00	0.00	1.00
Pancreas tumor	0.00	0.00	0.00	1.00
Liver Small	0.00	0.00	0.00	1.00

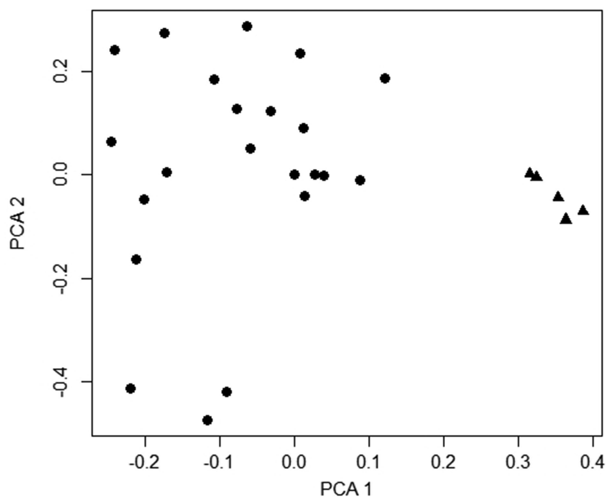


Figure 1. Cluster map of all the 22 variables. The dots represent the top 5 variables (triangles) and the remaining 17 variables (dots). The 5 significant variables cluster together.

RESULTS AND DISCUSSION

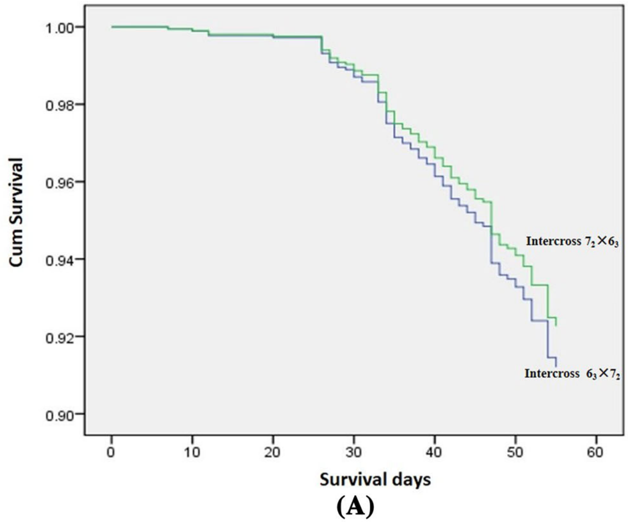
Principal Component Analysis Resulted in the Selection of 5 Categorical Variables

A total of 22 binary and ordinal variables were subjected to PCA to determine the relevance in relation to survival days. The 22 variables and the PCA statistics are listed in Table 1. About 5 of the 22 variables were deemed statistically relevant based on the dataset of this study. These were bursa atrophy, thymus atrophy, brachial, vagus, and sciatic nerve enlargement. These 5 variables were then included in the survival day analysis along with other variables. The number of survival days for MDV-challenged birds are reportedly affected by a few variables including, for example, the *B*-haplotypes of the major histocompatibility complex (MHC), sex, vaccination dosage, age at vaccination, intervals between vaccination and challenge, challenge virus dosage and pathogenicity (Martin et al., 1989; Baigent et al., 2007; Islam et al., 2007; Schat et al., 2013). The 5 variables identified with potential effects on survival days, which have not been systematically examined or reported before based on a search of the current literature, due to the observed top incidences, the clustered together feather in the PCA plot (Figure 1), and the correlation coefficients (Supplemental Table S1). There may be more variables contributing to survival days but these failed to be detected. This may be due to the low organ specific tumor incidences observed in this experiment.

Survival Analyses Detected no Difference in Survival Days Between the Reciprocal Crosses

Reciprocal cross, sex, MD and the 5 categorical variables, selected above based on the PCA results, were used as the dependent variables in the survival analyses. The cox-regression model was fitted twice: once with the dataset such that all the dependent variables, including bursa and thymus atrophy, brachial, vagus, and sciatic nerve enlargement, were coded as binary variables (0 = normal and 1 or 2 or 3 or 4 = abnormal) to minimize the number of observation levels for those variables, and to boost the frequency of observation for each observation level. The other fitting of the model was such that the observations of the 5 selected categorical variables remained as originally recorded (0, 1, 2, 3, or 4). None of the analyses revealed a significant difference in survival days between the 2 F_2 groups of the reciprocal crosses ($P > 0.05$, Figures 2A and 2B).

To further evaluate the finding above, a comparison for each of the traits, including all the categorical



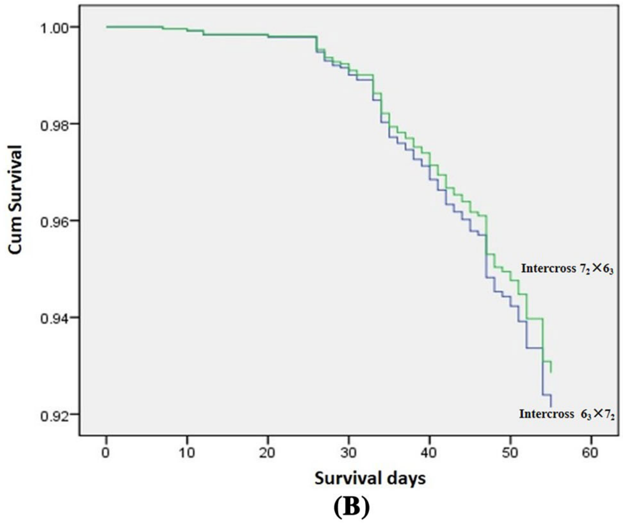


Figure 2. Survival plots showing survived day patterns between reciprocal crosses after Marek's disease virus (MDV) infection, (A) with 2 levels (normal and abnormal), (B) with the 5 major factors divided into 5 levels. The survival patterns showed the intercross $7_2 \times 6_3$ survived longer than intercross $6_3 \times 7_2$ but with no statistical significance ($P > 0.05$).

Table 2. Comparison between the 2 F_2 groups of the reciprocal crosses for each trait.

Variables	Reciprocal cross $6_3 \times 7_2$ (246)		Reciprocal cross $7_2 \times 6_3$ (224)		Chi-square P-value
Sex	124 (M)	122 (F)	115 (M)	109 (F)	0.840
Survival or mortality	54 (MT)	192 (S)	53 (MT)	171 (S)	0.659
MD or normal	117 (MD)	129 (N)	110 (MD)	114 (N)	0.738
	Tumor or abnormal	Normal	Tumor or abnormal	Normal	
Bursa atrophy	89	157	90	134	0.372
Thymus atrophy	95	151	95	129	0.403
Vagus	103	143	93	131	0.938
Brachial	82	164	70	154	0.630
Sciatic	63	183	62	162	0.612
Gonadal tumor	3	243	4	220	0.613
Liver tumor	14	232	17	207	0.408
Liver large	8	238	8	216	0.849
Liver medium	4	242	7	217	0.283
Liver Small	2	244	2	222	0.925
Liver focal	10	236	13	211	0.383
Liver diffusion	4	242	4	220	0.894
Spleen tumor	24	222	20	204	0.758
Spleen large	14	232	10	214	0.546
Spleen medium	10	236	9	215	0.979
Spleen focal	7	239	9	215	0.484
Spleen diffusion	17	229	11	213	0.360
Heart tumor	17	229	18	206	0.643
Lung tumor	2	244	5	219	0.205
Intestinal tumor	3	243	4	220	0.613
Pancreas tumor	4	242	3	221	0.798
Kidney tumor	6	240	7	217	0.651

Note: The numbers in brackets represent the number of individuals.
M means Male and F means Female.
S means Survival and MT means Mortality.
MD means Marek's Disease and N means normal.

variables, was conducted to examine difference between the reciprocal crosses by a chi-square test using the SAS package. No significant difference was detected for any of these traits ($P > 0.05$, Table 2). A final comparison for each of the 5 selected ordinal variables with their original scores was also conducted between 2 reciprocal crosses by crosstabs analysis and chi-square test using SAS. Again, no significant difference for any of those traits was detected ($P > 0.05$, Table 3).

The parental lines of the reciprocal crosses, the highly inbred lines 6_3 and 7_2, share a common major histocompatibility complex *B*-haplotype, the $B^{*}2$ haplotype, and yet 1 is known to be relatively resistance to MD and the other is highly susceptible (Bacon et al., 2000). The survival days of the 2 inbred line birds significantly differ in response to vv+ MDV challenge (Chang et al., 2012; Chang et al., 2014). The data from this study demonstrate that the survival days of the progeny of the line

Table 3. Comparison between the 2 F_2 groups of the reciprocal crosses for each of the 5 selected categorical traits with the original scores.

Variables	Levels	$6_3 \times 7_2$ (246)	$7_2 \times 6_3$ (224)	*P*-value
Bursa atrophy	0	157	134	0.680
	1	10	12	
	2	38	33	
	3	41	45	
	4	0	0	
Thymus atrophy	0	151	129	0.733
	1	10	7	
	2	25	26	
	3	60	62	
	4	0	0	
Vagus nerve enlargement	0	143	131	0.978
	1	11	11	
	2	76	66	
	3	15	16	
	4	1	0	
Brachial nerve enlargement	0	164	154	0.726
	1	18	12	
	2	51	43	
	3	12	14	
	4	1	1	
Sciatic nerve enlargement	0	183	162	0.674
	1	21	15	
	2	31	35	
	3	11	12	
	4	0	0	

6_3 and 7_2 reciprocal crosses did not differ from each other, suggesting that the grandparental (maternal or paternal) lineage has no or little effect on survival day characteristics.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

Table S1. The correlation coefficient of the 22 variables.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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